JOURNAL OF CHEMICAL EDUCATION
Cite This: J. Chem. Educ. 2018, 95, 1867−1871
Laboratory Experiment
pubs.acs.org/jchemeduc

Real-Time Visualization of *in Vitro* Transcription of a Fluorescent RNA Aptamer: An Experiment for the Upper-Division Undergraduate or First-Year Graduate Laboratory

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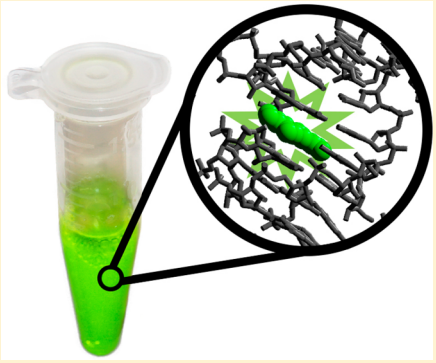
Supporting Information

ABSTRACT: A teaching laboratory experiment is described where students prepare *in vitro* transcription reactions of a fluorescent RNA aptamer, named Broccoli, and observe the production of the aptamer in real-time on a fluorescence plate reader. Alternate visualization methods with minimal costs are also described for laboratories lacking this instrumentation. Two optional experiments are also described. Optional Experiment 1 involves purification of RNA transcription reactions using a commercial spin column kit and having students correlate cleanup kit yield with transcribed aptamer fluorescence. Optional Experiment 2 involves running a polyacrylamide gel of the transcription reaction with a ladder, followed by staining with (*Z*)-4-(3′,5′-difluoro-4′-hydroxybenzylidene)-2-methyl-1-(2″,2″,2″-trifluoroethyl)-1*H*-imidazol-5-(4*H*)-one (DFHBI-1T) (selective for Broccoli) and a second stain with SYBR Gold (nonselective, allowing for simultaneous visualization of Broccoli and ladder). This experiment has the practical advantage of enabling aptamer visualization in laboratories without a fluorescence spectrometer or plate reader, as well as the pedagogical benefit of demonstrating specific activation of the fluorescence of a small molecule by an RNA aptamer in another context (gel staining). Each experiment allows students to perform straightforward, easily understood teaching laboratory experiments, including key concepts in cellular imaging, and RNA biochemistry widely employed in biochemical research.



KEYWORDS: *Upper-Division Undergraduate, Graduate Education/Research, Biochemistry, Hands-On Learning/Manipulatives, Electrophoresis, Fluorescence Spectroscopy, Nucleic Acids/DNA/RNA*

INTRODUCTION

The green fluorescent proteins (GFPs) from *Aequorea victoria* and *Renilla reniformis* are foundational tools in modern biochemistry, and they have experienced over 30 years of widespread use and development as reporters and protein markers.[1,2] A mainstay of biochemical research, they have been employed in undergraduate teaching laboratories, including several that have been described in this *Journal*.[3,4] In recent years, intense interest has developed in obtaining a corresponding tool for the fluorescent tracking of RNAs. Several such tools have been reported, of which perhaps the best known examples are named the Spinach and Broccoli RNA aptamers.[5,6] These aptamers bind to small-molecule analogues of the *p*-hydroxybenzylidene-imidazolidone (HBI) fluorophore formed in GFP by oxidative cyclization of the SYG sequence element (Figure 1a).[7] In the original Spinach selection, the difluoro analogue of this fluorophore, (*Z*)-4-(3′,5′-difluoro-4′-hydroxybenzylidene)-1,2-dimethyl-1*H*-imidazol-5-(4*H*)-one (DFHBI) (Figure 1b), was employed, with the difluoro substituents providing electron-withdrawing character, which stabilized the phenolate conjugate base of DFHBI; the phenolate form of the fluorophore was observed in enhanced GFPs (eGFPs) as well.[1,5,8] The same group that selected the Spinach and Broccoli aptamers later developed

Received: September 22, 2017
Revised: July 16, 2018
Published: August 15, 2018

DOI: 10.1021/acs.jchemed.7b00735
J. Chem. Educ. 2018, 95, 1867−1871

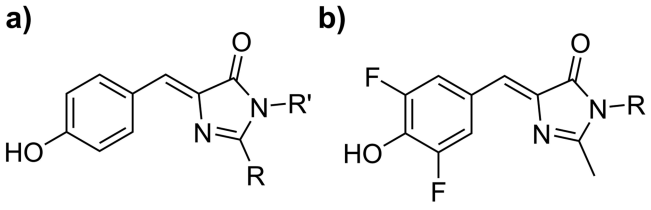


Figure 1. Fluorophores from *Aequorea* and *Renilla* GFPs (a; R = N-terminal GFP fragment, R′ = C-terminal GFP fragment) and Spinach and Broccoli aptamers (b; DFHBI; R = Me; DFHBI-1T, R = CH_2CF_3).

(*Z*)-4-(3′,5′-difluoro-4′-hydroxybenzylidene)-2-methyl-1-(2″,2″,2″-trifluoroethyl)-1*H*-imidazol-5-(4*H*)-one DFHBI-1T (Figure 1b), in which the methyl substituent of DFHBI was modified to trifluoroethyl; this change provided a spectral shift, thus allowing the ligand–aptamer complex to be better aligned with the wavelengths found in a microscopy filtercube for GFPs.[9] The original Spinach aptamer has gone through several rounds of iteration, including a better-folding "Spinach2", minimization to "Baby Spinach" in a paper describing its crystal structure and, recently, "Broccoli", which was selected by fluorescent sorting of a population of live bacterial cells expressing a DNA library coding for RNA aptamers after several rounds of *in vitro* selection.[6,10,11] The Spinach/Broccoli family of aptamers is known to be compatible with real-time kinetic monitoring of transcription by fluorescence, as has been shown in several systems.[12−14] The Broccoli aptamer was thus employed.[6]

A laboratory experiment involving fluorescence detection of GFP was previously described in this *Journal*, as well as experiments involving *in vitro* RNA transcription of another RNA (the hammerhead ribozyme).[3,4,15−17] To our knowledge, no classroom exercises involving fluorescent RNA aptamers have been reported to date. This laboratory experiment was performed with a cohort of incoming graduate students, with the pedagogical goal of enhancing the understanding, comfort, and confidence of students in theory and techniques related to *in vitro* synthesis of RNA and fluorescent visualization of RNA. This laboratory experiment was also performed with a cohort of undergraduate chemical biology students, presenting the aptamer along with a green fluorescent protein, with the goals of educating students in fluorescent visualization of biopolymers generally, as well as the aforementioned RNA biology techniques.

MATERIALS AND METHODS

Equipment

The transcription reaction was visualized in real-time using a plate reader with its associated software packages, scanning the plate every 5 min to obtain kinetic data. Any fluorescence plate reader capable of detecting the commonly employed GFP or fluorescein wavelengths is suitable. With the graduate cohorts, a monochromator-based plate reader (Molecular Devices Gemini EM) was used with an excitation wavelength of 472 nm and an emission wavelength of 507 nm. With the undergraduate cohort, a different filter-based plate reader (BioTek Synergy HTX) was used with 485 nm excitation and 528 nm filters. Assays were performed in 384-well plates at 20 μL reaction volume, with pairs of students sharing a reaction. A fluorescence spectrometer (either cuvette or NanoDrop/Qubit type) is also suitable for end point readings. Visual methods of aptamer visualization as a supplemental method or an inexpensive alternative accessible to laboratories lacking quantitative fluorescence information are also described (Supporting Information, Figure S1, p S7).

In Optional Experiment 1, quantification of samples after cleanup was performed on a UV–vis spectrophotometer. In Optional Experiment 2, polyacrylamide gel electrophoresis (PAGE) analysis was performed with staining and visualization on a gel documentation system.

Chemicals

The sources of the chemicals and Broccoli DNA template can be found in the Supporting Information. T7 RNA polymerase expressed in-house and commercial (NEB) T7 RNA polymerase are both suitable (Supporting Information). The Broccoli template is typically prepared as a synthetic Ultramer oligonucleotide from IDT; alternately, the Broccoli template outside the F30 scaffold suffices for transcription. For the shorter, non-F30-Broccoli, the sense strand, d(**TAA TAC GAC TCA CTA TAG** GAG ACG GTC GGG TCC AGA TAT TCG TAT CTG TCG AGT AGA GTG TGG GCT C) (T7 promoter in bold) forms a duplex with its complement and is transcribed by T7 RNA polymerase to the Broccoli RNA r(GGA GAC GGU CGG GUC CAG AUA UUC GUA UCU GUC GAG UAG AGU GUG GGC UC). The F30-Broccoli template sequence is given in the Supporting Information. The cleanup of the RNA transcription products was performed using the Zymo Research RNA Clean and Concentrator-25 Kit. This kit was supplemented with Turbo DNase. The reagents used for PAGE can either be purchased as a Mini-PROTEAN kit with precast gels, or made in-house depending on time and budget. For recipes of buffers and reagent prepared in-house, see the Supporting Information.

Experimental Procedures

The main experiment involves the transcription of a Broccoli aptamer from a DNA template using T7 RNA polymerase. *In vitro* transcription reactions with T7 RNA polymerase require, at minimum: (1) a DNA template coding for the RNA of interest, with a double-stranded promoter region for T7 RNA polymerase, (2) T7 RNA polymerase, (3) r(NTPs), and (4) a suitable buffer.[18] The transcription reactions contain these components, as well as (5) DFHBI-1T, the small-molecule ligand that becomes fluorescent upon binding to the transcribed aptamer, and inorganic pyrophosphatase.

Inorganic pyrophosphatase hydrolyzes the pyrophosphate leaving group produced by incorporation of an NTP into a nascent strand of RNA, producing two phosphate ions. The inclusion of this enzyme in the transcription mixture prevents the buildup of pyrophosphate, which otherwise would chelate Mg^{2+} ions that are required both for transcription and folding of the produced aptamer. As negative controls, teaching assistants (TAs) prepared two samples: one in which the DNA template was omitted, resulting in no aptamer production and no fluorescence enhancement; and one in which the pyrophosphatase was omitted, resulting in diminished aptamer production, lower fluorescence enhancement, and visible precipitation of magnesium pyrophosphate. A sample containing fluorescein as a fluorescence standard was also included.

When possible, all RNA work begins with RNase decontamination solution treatment, with students beginning by sterilizing their working area. However, given the short transcript length (ca. 50–150 nt depending whether Broccoli or F30-Broccoli is employed) and stable fold of the RNA used

DOI: 10.1021/acs.jchemed.7b00735
J. Chem. Educ. 2018, 95, 1867−1871

in this laboratory experiment, we have found the use of RNase decontamination is not critical.

Students prepared reactions in pairs in PCR or 1.5 mL Eppendorf tubes. The completed reaction mixture was transferred into wells of a 384-well assay plate. The reaction is typically performed in 20 μL reaction volumes. However, 10 μL volumes are suitable if it is desired to use lower quantities of reagents (Figure S2, p S8). Using either commercial or in-house overexpressed T7 RNA polymerase is suitable (Figure S3, p S9). The 384-well plate was monitored in a fluorescence plate reader to obtain kinetics readings of the reaction progression at 5 min intervals.

Further experimental details can be found in the Supporting Information, including information on Optional Experiment 1 and Optional Experiment 2 (Figures S4 and S5, pp S10–S16).

HAZARDS

The *in vitro* transcription reaction employs minimally hazardous materials. Tris base, magnesium chloride, and potassium chloride are irritants. Spermidine trihydrochloride is a potential sensitizer. DTT is an irritant and toxic. DFHBI has no known hazards. Accordingly, good laboratory practice should be employed, including any specific requirements of the instructor's institution for handling of synthetic DNA. The optional electrophoresis module employs acrylamide (neurotoxic as the monomer), TEMED (corrosive, flammable), ammonium persulfate (irritant, sensitizer, oxidizer), and SYBR Gold (not mutagenic by the Ames test, but good laboratory practices should be observed) 10,000× solution in DMSO (irritant, flammable). If a UV light is used, appropriate precautions (eye and skin protection) should be used. The optional purification module presents no special hazards except the use of ethanol, which is flammable. Refer to reagent-specific material safety data sheets for further information. All reagents should be handled with gloves and eye protection.

RESULTS AND DISCUSSION

The results of the primary transcription experiment will vary depending on the visualization equipment employed. A plate reader will provide quantitative fluorescence data, while photographic measurement will provide more qualitative data (Figures 2 and 3). The level of fluorescence is variable even among successful reactions when prepared by the same individual; one likely source of variability is the inherent margin of error associated with pipetting 1–2 μL volumes. In the event of a reaction that fails completely, a troubleshooting section is included in the Supporting Information.

This laboratory experiment was performed three times under conditions where no special precautions were taken to avoid RNase contamination, including with undergraduates. The graduate cohorts performed the laboratory experiment at a student retreat at a remote site in northern Minnesota where it is essentially impossible to obtain RNase-free conditions given time constraints and the other laboratories sharing the space during the period in which this exercise was performed, while the undergraduate cohort performed the laboratory experiment in a general-use chemical biology teaching laboratory. Most students typically succeed at producing fluorescent aptamer. For example, in the April 2018 iteration of this laboratory experiment with undergraduates, all but two pairs (93% of students) among 30 pairs that performed the laboratory experiment obtained detectable fluorescence above background, and all but six pairs (80% of students) obtained at least 40% of the highest fluorescence value obtained in the class. The trace in Figure 2 shows data from a student sample closest to the median fluorescence value (55% of the highest fluorescence value obtained in the class).

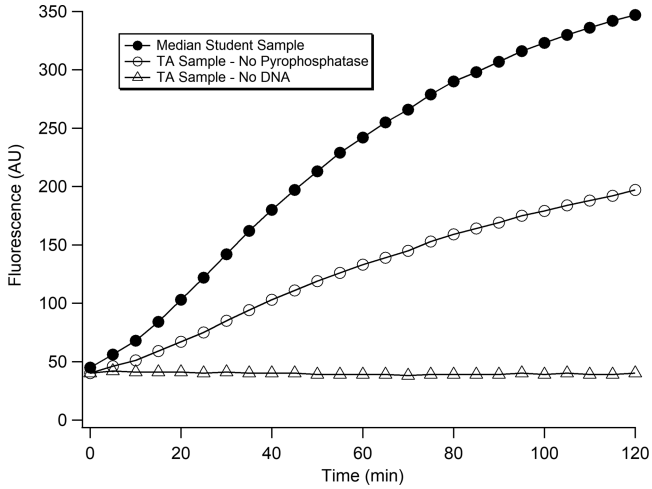


Figure 2. Real-time traces of student fluorescence reading during the transcription of Broccoli aptamer in a fluorescence plate reader, as well as standard reactions prepared by TAs omitting pyrophosphatase or DNA template. Data were collected at 5 min time intervals and are shown as a continuous connected line to guide the eye.

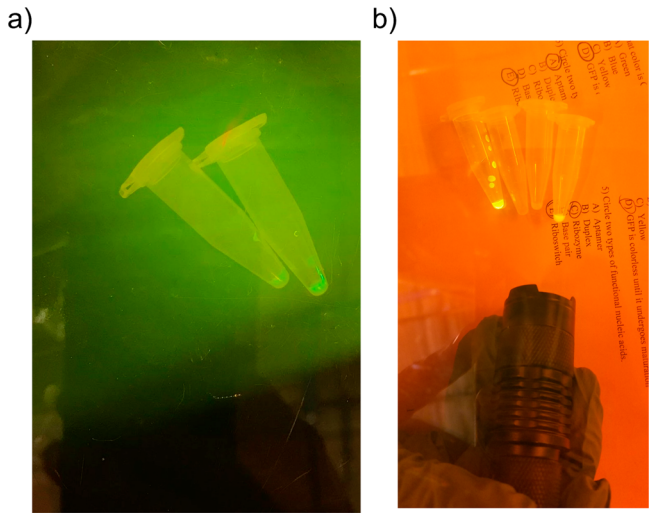


Figure 3. Student-collected images from the April 2018 undergraduate laboratory experiment using a blue LED flashlight for illumination. Students successfully used an inexpensive transparent orange clipboard (panel a, left to right): student sample, fluorescein standard. They also used an orange filter for a blue LED gel box (panel b, left to right): fluorescein standard, no DNA control, no pyrophosphatase control, student sample. The image illustrates that the less-expensive clipboard filter is suitable for observation, albeit with increased background fluorescence.

As an alternative means of observing the success or failure of aptamer transcription reactions in laboratories lacking quantitative fluorescence instrumentation, or simply as a more visually direct way of observing experimental results, transcription samples were observed directly in PCR tubes, using four different types of equipment commonly available in teaching laboratories or available at very low cost: a blue-light gel transilluminator with an orange filter (Figure S1a,b, p S7), a

DOI: 10.1021/acs.jchemed.7b00735
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gel documentation system (Figure S1c,d), a UV lamp (Figure S1e,f), and a blue LED flashlight with the filter from the gel transilluminator, or an orange transparent plastic clipboard that is commercially available for ca. $15 (Figure 3, Figure S1g,h). Each method allows visualization of fluorescent aptamer, demonstrating that these reactions can be characterized with little or no capital and equipment required. Except for the photos from the gel documentation system in Figure S1c,d, all photographs in Figure S1 were collected using a commercial cell phone camera (Motorola Droid Turbo 2).

In the August 2017 student retreat and April 2018 undergraduate laboratory experiment, the orange filter/LED flashlight method shown in Figure 3 and Figure S1g-h was employed. Student results from the April 2018 laboratory experiment are shown in Figure 3. In postlab attitudinal surveys administered to the August 2017 cohort, multiple students commented that they appreciated the visual nature of this experiment. The clipboard, which blocks blue light (from the excitation source, the flashlight) and passes green light (from the emission source, the students' samples), also provides an opportunity for instructors to discuss the use of filters in, for example, fluorescence microscopy. Given students' appreciation of this mode of analysis, we intend to continue to use it in conjunction with the more quantitative data collected from the fluorescence plate reader. We believe other instructors will find these techniques to be complementary as well.

SUMMARY

This experiment was performed three times. It was performed twice by two cohorts of 20 and 24 first-year graduate students in the University of Minnesota Molecular, Cellular and Structural Biology (MCSB) graduate program, a joint program for first-year graduate students in the Biochemistry, Molecular Biology and Biophysics (BMBB) and Molecular, Cellular, Developmental Biology and Genetics (MCDB&G) graduate programs. The students performed this exercise in pairs at the new graduate student retreat at the Itasca Biological Station and Laboratories in August 2016 and August 2017. A pre- and postmodule attitudinal survey was administered to course participants in the 2017 iteration of this laboratory experiment: questions relating to understanding of a selection of RNA-related terms went from 2.2 to 3.6 on a 1−4 scale; comfort in performing RNA-related techniques went from 3.7 to 4.4 on a 1−5 scale; and confidence in performing RNA-related experiments went from 3.3 to 4.4 on a 1−5 scale. Details of the survey questions and anonymized student responses (student identifiers hashed with SHA3-256) from the 2017 cohort are provided in the Supporting Information. It was performed an additional time by a cohort of 60 upper-division undergraduates in the University of Minnesota Foundations of Chemical Biology Laboratory. A pre- and postlab quiz relating to the fluorescent proteins that inspired the development of the Broccoli aptamer and functional nucleic acids was administered to this audience. Scores had a mean of 2.6/5 (prelab) and 3.9/5 (postlab). Details of the quiz and pre- and postlab score distributions are provided in the Supporting Information (Figures S6 and S7, p S36).

As is typical for the MCSB program, this cohort of students came largely from undergraduate programs in biochemistry and biology, with a number of incoming students from other related undergraduate programs, such as chemistry, clinical laboratory sciences, and biotechnology. The undergraduate cohort was an audience of chemistry majors. Novice experimentalists at the undergraduate level and higher are generally able to successfully perform this protocol. This experiment is also routinely used in our research laboratory as a training exercise for new personnel in the handling of RNA, performing *in vitro* transcription, performing cleanup of RNA, and performing polyacrylamide gel electrophoresis.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available on the ACS Publications website at DOI: 10.1021/acs.jchemed.7b00735.

Procedures for primary experiment and Optional Experiments 1 and 2, troubleshooting guide, recipes for reagents, supplies with part number and costs (as of 2017), attitudinal survey questions (PDF, DOCX)

Attitudinal survey results (XLSX)

Transcription analysis template for well plate (XLSX)

Spreadsheet with calculations for TA and students (XLSX)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by University of Minnesota startup funds to A.E.E. and K.P.A., and University of Minnesota Department of Chemistry teaching funds for E.E.C. and J.D.S. Experiments performed at the Itasca retreats were supported by the MCSB graduate program at the University of Minnesota. We gratefully acknowledge the assistance of the University of Minnesota faculty, staff, and teaching assistants involved in the planning and execution of the laboratories in which these experiments were performed, particularly Ian Armitage, Taysir Bader, Huarui Cui, Michael Freeman, Michael Jarcho, Sue Knoblauch, Andrew Marshall, Megan McCarthy, Michael Pickett-Leonard, Barbara Pinch, Taylor Reid, Amber Schoenecker, Shabnam Sharifzadeh, Margaret Titus, and Yiao Wang. We thank members of the Engelhart and Adamala laboratories and Mary Peek (Georgia Institute of Technology) for helpful comments.

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